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I, ANNA MAIJA EVERETT, ACTING TEAM LEADER EXAMINATION SUPPORT & SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 1361 for a patent by STEM CELL SCIENCES PTY LTD filed on 30 June 1999.

WITNESS my hand this
Twenty-second day of May 2000

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PROVISIONAL SPECIFICATION

Invention Title: **Nuclear addition II**

The invention is described in the following statement:

NUCLEAR ADDITION II

This invention relates to the generation of cells, embryos and animals by nuclear addition, including but not limited to the generation of cloned and transgenic animals.

Nuclear transfer is the replacement of the nucleus of one cell with that of another. The ability to produce live offspring by nuclear transfer is an objective which has been sought for some time by animal breeders. The ability to produce cloned offspring in such a manner would enable the production of large numbers of identical offspring and the ability to genetically modify and/or select cell populations of the required genotype (e.g. sex or transgenic) prior to embryo reconstruction.

Whilst nuclear transfer has been described in some animals, the procedures used are often inefficient and have not yet been successfully applied to many species.

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In a first aspect of the present invention, there is provided a method of preparing an aneuploid cell which method includes

providing

a donor nucleus, and

a recipient cell; and

adding the donor nucleus to the recipient cell to produce an aneuploid cell.

In a second aspect of the present invention there is provided a method of generating an animal embryo which method includes

providing

a donor nucleus, and

a recipient cell;

introducing the donor nucleus into the recipient cell to produce an aneuploid cell; and

generating an animal embryo from the aneuploid cell.

Accordingly, the method of the present invention involves nuclear addition, ie. the addition of the nucleus of one cell to another cell.

Whilst Applicant does not wish to be restricted by theory, it is postulated that the donor nucleus forms the animal embryo and an aneuploid placenta may be formed. This may provide advantages over normal diploid placentas. For example, the aneuploid placenta may be bigger and more vigorous, which may in turn improve embryo viability. Alternatively the nucleus of the recipient cell may be expelled from the cell and participate no further in the development of the embryo.

The process of the present invention may include the further step of generating an animal from the animal embryo.

In a further aspect of the present invention there is provided a method of preparing a genetically modified aneuploid cell, said method including

providing

a donor nucleus which has been genetically modified to eliminate or reduce an undesirable activity or to provide for, or increase, a desirable activity, and

a recipient cell; and

transferring the donor nucleus to the recipient cell to produce the genetically modified aneuploid cell.

The donor nucleus may be modified by conventional means or may comprise the combination of a donor nucleus and an exogenous nucleic acid molecule of the type described in Australian provisional patent application PQ0202, the entire disclosure of which is incorporated herein by reference.

In a still further aspect of the present invention there is provided a method

of generating a transgenic animal embryo said method including

providing

a donor nucleus which has been genetically modified to eliminate or reduce an undesirable activity or to provide for, or increase, a desirable activity, and

a recipient cell;

adding the donor nucleus to the recipient cell to produce a genetically modified aneuploid cell; and

generating a transgenic animal embryo from said aneuploid cell.

In a preferred aspect of the present invention, the method may include subjecting the DNA of the recipient cell to an inhibition step.

Inhibition, e.g removal or destruction of the DNA, is preferred to reduce or eliminate co-mingling of the recipient cell DNA with the introduced DNA. This is illustrated in Figure 1 hereto. Embryos so formed may not be clones, and may, in turn, not develop to full-term.

In one form of this aspect of the present invention, the method may include the further step of

removing or destroying the DNA of the recipient cell, preferably prior to division of the aneuploid cell.

Removal of the DNA of the recipient cell may be performed, for example, approximately 1 to 20, more preferably approximately 3 to 12, most preferably approximately 6 to 8 hours after completion of the nuclear addition procedure, and prior to first embryo cleavage. Destruction of the DNA may be achieved utilising chemical or laser microsurgical techniques or the like.

In an alternate form, the method includes the further steps of

providing a co-mingling inhibitor, and

subjecting the aneuploid cell to a co-mingling inhibitor for a period sufficient to reduce or eliminate co-mingling of the recipient cell DNA and introduced DNA.

The co-mingling inhibitor may be a cytoskeletal (cytokinesis) or karyokinesis

Cytochalasin B, Colcemid, Taxol or Nocodazole. The aneuploid cell may be exposed to the co-mingling inhibitor for a period, up to pronuclear formation, of for example approximately 1 to 6 hours after reconstruction and preferably prior to normal embryo cleavage.

Accordingly, in a preferred embodiment of the present invention, there is provided a method of generating an animal embryo which method includes

providing

a donor nucleus; and

a non-enucleated recipient cell;

transferring the donor nucleus into the recipient cell to produce an aneuploid cell;

subjecting the recipient cell to an activation step, prior to, concomitant with, or subsequent to introducing the donor nucleus; and

optionally subjecting the DNA of the recipient cell to an inhibition step.

In a preferred aspect of the present invention the method may include the further step of

maintaining the aneuploid cell in a suitable medium for a period sufficient to allow the cell to recover a substantially normal shape, prior to activation.

Applicant has discovered that the number of viable embryos produced may be significantly increased by permitting the reconstituted cell to be maintained in a quiescent state for a period sufficient to allow the cell to recover a substantially normal, e.g. generally circular, shape. The quiescent step may be undertaken in a manner similar to that described in Australian provisional patent application PQ0204, to applicants, the entire disclosure of which is incorporated herein by reference.

Whilst applicant does not wish to be restricted by theory, it is postulated that the quiescent period permits the cell to return to a more normal state after which cell fusion may proceed more efficiently.

The reconstituted cell may be maintained in a suitable medium preferably for a period of approximately 3 to 8 hours, more preferably approximately 4.5 to 6 hours. It is desirable, however, that the quiescent period end before any, or any substantive division, ensues.

In a preferred embodiment of the present invention, the method may include the preliminary step of

subjecting the recipient cell to an activation step, prior to transfer of the donor nucleus.

Activation occurs during fertilisation when the penetrating sperm triggers the resumption of meiosis. Activation is characterised by calcium oscillation, release of cortical granules, extrusion of the second polar body, pronuclear formation and ultimately cleavage. The aneuploid cell may be treated with, but not limited to, for example, ethanol, calcium ionophore or electrical stimulation to induce activation. Activation may be performed prior to, concomitantly with, or after, the addition of the donor nucleus. Activation is preferably performed prior to the addition of the donor nucleus.

Applicants have found, in this preferred embodiment, improved results where a preliminary activation step is undertaken.

Preferably the aneuploid cell is subsequently subjected to cell fusion.

Where the preferred preliminary activation step is not utilised the aneuploid cell may be subjected to a cell fusion/activation step. For example, where electrical pulses are utilised for cell fusion, the voltage may be selected to simultaneously initiate activation.

The aneuploid cell may be also be subjected to simultaneous cell fusion/activation or a process of cell fusion followed later by activation.

The method according to this aspect of the present invention may include the further step of generating a transgenic animal from the transgenic animal

embryo.

The donor nucleus may be of any suitable type and from any suitable species. The donor nucleus may be contained in a karyoplast or cell. The donor nucleus may be of embryonic, embryonal tumor, foetal or adult origin. Donor nuclei may be prepared by removing the nucleus and a portion of the cytoplasm and plasma membrane surrounding it from early pre-implantation stage embryos (for example zygotes, 4- to 16- cell embryos) for example using microsurgery. When nuclei from more advanced embryonic cells are used the whole blastomere may be transferred to the recipient cytoplasm. Embryonic or foetal fibroblasts may be used. Embryonic stem (ES) cells [isolated from inner cell mass (ICM) cells, embryonic disc (ED) cells or primordial germ cells (PGC)] may be used. A cell line derived from an embryonal tumor may be used (eg. embryonal carcinoma (EC) or yolk sac tumor cells). Adult cells such as fibroblasts may also be used. In this case the whole cell may be fused to the recipient cytoplasm.

It is particularly preferred that the donor cells be at a particular stage in the cell cycle, for example G_0, G_1 or S-phase. Applicant has found that it is possible to isolate populations of cells which are enriched for cells at each stage in the cell cycle by sorting the cells on the basis of size, for example using FACS. This avoids the use of stains, which are toxic to the cells. Staining can be used on a sample of each size-sorted population to identify what stage in the cell cycle that population is at.

The recipient cell may be of any suitable type and from any suitable species. Recipient cells may be *in vivo* or *in vitro* produced oocytes. Recipient cells may be oocytes. Metaphase I (MI) oocytes or Metaphase II (MII) oocytes may be used. Other sources of recipient cells include zygotes, fertilised oocytes, 2-cell blastomeres, cell lines produced from gonads, or any cell type suitable for allowing the successful addition of a nucleus. Oocytes, for example arrested in the second metaphase of meiotic maturation (MII oocytes) are preferred.

The donor nucleus may be transferred to the recipient cell by any suitable method. Such methods include, but are not limited to, microsurgical injection, and

cell fusion for example mediated by electrical pulses (electrofusion), chemical reagents such as polyethyleneglycol or the use of inactivated virus such as Sendai virus.

Preferably the donor nucleus for addition is introduced under the zona pellucida.

Optional removal of the DNA from the recipient cell may be performed by any suitable technique. The DNA may be removed by microsurgery. Alternatively, nonphysical approaches such as inactivation of the chromosomes by UV, chemicals or laser irradiation may be used.

In a preferred embodiment of this aspect of the present invention, the donor nucleus for addition may be from an embryo that is itself the product of nuclear transfer or nuclear addition. This could be called serial nuclear transfer and/or addition.

Serial nuclear transfer and/or addition may improve the capacity of differentiated nuclei to direct normal development. Whilst applicant does not wish to be restricted by theory, serial nuclear transfer and/or addition is postulated to improve the developmental capacity of transplanted nuclei by allowing specific molecular components in the oocyte to assist in chromatin remodelling that is essential for nuclear reprogramming. Serial nuclear transfer and/or addition is not restricted to a singular event but may be initiated on more than one occasion to sequentially improve conditions for chromatin remodelling, nuclear reprogramming and embryonic development.

In a further aspect of the present invention, applicants have discovered it is possible to utilise donor cells or nuclei derived from differentiated sources, e.g. from skin, neural or liver tissue, and use those cells to generate cells of an alternate type.

In this aspect, it is necessary to undertake a preliminary cell partial de-differentiation step.

Accordingly, in this aspect, the present invention provides a method of [illegible]

providing

a donor nucleus or cells from a differentiated source; and

a non-enucleated recipient cell;

transferring the donor nucleus or cells into the recipient cell to produce a first aneuploid cell;

subjecting the recipient cell to an activation step, prior to, concomitant with, or subsequent to introducing the donor nucleus or cells;

culturing the aneuploid cell for a period sufficient to permit partial de-differentiation;

isolating the partially de-differentiated nucleus or cell; and

transferring the de-differentiated nucleus or cell into a second recipient cell to produce a second aneuploid cell.

The second aneuploid cell may be further processed as described above.

The partial de-differentiation may be conducted in a manner similar to that described in Australian provisional patent application PQ0201, to applicants, the entire disclosure of which is incorporated herein by reference.

An animal embryo may be generated from the aneuploid cell by any suitable method. Embryonic development may be initially *in vitro* and subsequently in a surrogate. Thus, the reconstituted cell may be initially cultured *in vitro* to produce an embryo and then the embryo may be transferred to a surrogate for subsequent development into an animal. *In vitro* culture of the aneuploid cells may be in any suitable medium.

The animal embryo or animal may be of any type, and includes bird, fish, reptile and mammalian (including ungulate and primate) embryos including human embryos, e.g. murine, bovine, ovine or porcine embryos. Preferably, the animal embryo is a porcine embryo, bovine embryo, sheep embryo, murine embryo or human embryo.

The donor nucleus for addition and recipient cell which are used in the method of the present invention may be of any suitable origin. Preferably they are [illegible] bovine, ovine, rodent, avian, fish, reptile, murine or human origin.

The method of the present invention may be used to generate transgenic animals. For example, a new gene may be expressed and/or an existing gene may be deleted in the transgenic animal. The addition of new genes is technically less demanding than the deletion of existing genes.

As used in this specification the term "transgenic", in relation to animals and all other species, should not be taken to be limited to referring to animals containing in their germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line has been the subject of prior technical intervention by recombinant DNA technology particularly nuclear addition. So, for example, an animal in whose germ line an endogenous gene has been deleted or modified (either by modifying the gene product or pattern of expression) is a transgenic animal for the purposes of this invention, as much as an animal to whose germ line an exogenous nucleic acid sequence has been added.

The donor nucleus used for nuclear addition may be genetically modified by modifying, deleting or adding one or more genes. The gene(s) to be modified, deleted or added may be of any suitable type.

The process of modifying a gene may involve the introduction of one or more mutations in both copies of the target gene. Suitable cells may take up the mutation(s) and then be used to generate an animal. One copy of the gene may be disrupted in the cell and the resultant heterozygous animals bred with each other until one with both copies of the gene mutated is found. Alternatively, both copies of the gene may be modified *in vitro*.

To target an endogenous gene rather than introduce random mutations, a DNA construct (transgene) including a nucleic acid sequence which is

substantially isogenic to at least one or more portions of the target gene except for

The targeting DNA may comprise a sequence in which the desired sequence modifications are flanked by DNA substantially isogenic with a corresponding target sequence in the genome to be modified. The substantially isogenic sequence is preferably at least about 97-98% identical with the corresponding target sequence (except for the desired sequence modifications), more preferably at least about 99.0-99.5% identical, most preferably about 99.6% to 99.9% identical. The targeting DNA and the target DNA preferably share stretches of DNA at least about 75 base pairs that are perfectly identical, more preferably at least about 150 base pairs that are perfectly identical, even more preferably at least about 500 base pairs that are perfectly identical. Accordingly, it is preferable to use targeting DNA derived from cells as closely related as possible to the cell being targeted; more preferably, the targeting DNA is derived from cells of the same haplotype as the cells being targeted. Most preferably, the targeting DNA is derived from cells of the same individual (or animal) as the cells being targeted. Preferably, the targeting DNA sequence comprises at least about 100-200 base pairs of substantially isogenic DNA, more preferably at least about 300-1000 base pairs of substantially isogenic DNA, even more preferably at least 1000-15000 base pairs of substantially isogenic DNA.

As used herein, the term isogenic or substantially isogenic DNA refers to DNA having a sequence that is identical with or nearly identical with a reference DNA sequence. Indication that two sequences are isogenic is that they will hybridise with each other under the most stringent hybridisation conditions (see e.g., Sambrook, J., Fritsch, E.F., Maniatis, T., (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbour Laboratory Press, New York); and will not exhibit sequence polymorphism (i.e. they will not have different sites for cleavage by restriction endonucleases). The term "substantially isogenic" refers to DNA that is at least about 97-99% identical with the reference DNA sequence, and preferably at least about 99.5-99.9% identical with the reference DNA sequence and in certain cases 100% identical with the reference DNA sequence. Indications

that two sequences are substantially isogenic is that they will still hybridise with each other under the most stringent conditions (see Sambrook, J. et al. 1989) and that they will only rarely exhibit restriction fragment length polymorphism (RFLP) or sequence polymorphism (relative to the number that would be statistically expected for sequences of their particular length which share at least about 97-98% sequence identity). In general, a targeting DNA sequence and a host cell sequence are compared over a window of at least about 75 consecutive nucleotides. DNA sequences compared between individuals of a highly inbred strain, such as the MHC inbred miniswine, are generally considered to be substantially isogenic even if detailed DNA sequence information is not available, if the sequence do not exhibit sequence polymorphisms by RFLP analysis.

Thus, the donor nucleus for addition may be genetically modified by modifying an endogenous gene in the donor nucleus. The endogenous gene may be modified by introducing into said donor nucleus a DNA construct including a nucleic acid sequence which is substantially isogenic to at least one or more portions of the endogenous gene and includes one or more mutations, such that there is homologous recombination between the DNA construct and the endogenous gene.

The introduction of new genetic material and the subsequent selection of cells harbouring the desired targeted integration requires expansion and clonal selection of each founder transgenic cell. A limitation to applying this process in nuclear transplantation programs is the number of cell divisions which the transfected cell must undergo to provide sufficient material for molecular analysis of each transgenic colony and subsequent supply of nuclei for transfer. The great majority of cells suitable for *in vitro* genetic modification and subsequent nuclear transfer have limited *in vitro* propagation capacity. It is therefore desirable to utilise transfection and selection systems which generate and/or identify correctly targeted clones at high efficiency and with limited requirement for *in vitro* propagation.

A particularly efficient approach to selecting for correctly targeted clones is to use IRES gene trap targeting vectors, as described in Australian Patent

678234, the entire disclosure of which is incorporated herein by reference. The [illegible] (TAA_3) IRES-lacZ, (TAA_3) IRES-lacZ lox neo-tk lox, (TAG_3) IRES-lacZ/mcIneo, SA lacZ-IRES neo, SA (TAA_3) IRES-nuclear lacZ, SA (TAA_3) IRES-nuclear lacZ lox Gprt lox, IRES-βgeo, (TAA_3) IRES-βgeo, SA IRES-βgeo SA Optimised IRES-β geo, IRES-nuclear βgeo, SA IRES-nuclear βgeo, SA (TAA_3) IRES-nuclear βgeo, SA Optimised IRES-nuclear βgeo, IRES-zeo, SA IRES-zeo, IRES-hph, SA IRES-hph, IRES-hph-tk, IRES-bsd, SA IRES-bsd, IRES-puro. IRES gene trap targeting vectors provide a significant enhancement in gene targeting efficiency by eliminating a large proportion of random integration events. IRES gene trap targeting vectors rely upon functional integration into an actively transcribed gene (such as the target gene) for expression of the selectable marker. Random integrations into non-transcribed regions of the genome are not selected.

In a preferred embodiment, it may be desirable to remove the selectable marker cassette from the targeted locus to eliminate expression of the eg. antibiotic resistance gene. One approach is to flank the IRES selectable marker cassette with suitable DNA sequences which act as recombination sites following the addition of a suitable site-specific recombinase. One example of a suitable recombinase site is the lox site which is specific for the Cre recombinase protein. Another example of a suitable recombinase is the FLP/FRT recombinase system (O'Gorman, S., Fox, D.T., Wahl, G.M. (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* 251(4999), 1351-5).

High efficiency gene targeting and selection has a significant advantage in that suitably stringent selection systems, such as the IRES gene trap targeting vectors, can eliminate the need for biochemical analysis of clonal cell lines. In this instance, individual nuclei from a pool of uncharacterised transgenic cells should generate offspring of the desired phenotype at a ratio equivalent to the selected pool. The elimination of clonal selection may be particularly useful where only limited *in vitro* propagation is desirable or possible. One such instance includes the culture of embryonic nuclei for nuclear transfer. Embryonic nuclei are more

efficient than latter stage somatic cells for generating live born offspring by nuclear transfer. However, totipotential embryonic cells can not be cultured for extended periods for any other species than mice. Nuclear recycling of embryonic nuclei provides an opportunity to maintain, expand and genetically manipulate multipotential cells from animals *in vitro*.

The DNA constructs may be engineered in bacteria and then introduced into the cells. The transgenes may be introduced into the cells by any suitable method. Preferred methods include direct injection, electroporation, liposomes or calcium phosphate precipitation. Direct injection is the preferred method for embryonic cells while electroporation is more suitable for embryonic fibroblast and embryonic stem cell cultures.

Whilst applicant does not wish to be restricted by theory, it is thought that regions of substantially isogenic DNA either side of the mutation drag the transgene to the target site where it recombines and introduces the mutation. It is further thought that the main contributing factor for increasing the efficiency of introducing a specific mutation in a given gene is the degree of similarity between the target DNA and the introduced DNA. Thus, it is preferred that the DNA is isogenic (genetically identical) not allogenic (genetically dissimilar) at the genetic locus that is to be targeted.

In a further aspect of the present invention there is provided an aneuploid animal cell or modified aneuploid animal cell produced by the methods of the present invention. Preferably the aneuploid animal cell or modified aneuploid animal cell is a porcine, murine, ovine, bovine, caprine or human cell.

In a further aspect of the present invention there is provided an animal embryo or transgenic animal embryo produced by the methods of the present invention. Preferably the animal embryo or transgenic animal embryo is a porcine, murine, ovine, bovine, caprine or human embryo.

In a still further aspect of the present invention there is provided an animal or transgenic animal produced by the methods of the present invention. Preferably

the animal or transgenic animal is a porcine, murine, ovine, bovine, caprine or

The present invention will now be more fully described with reference to the accompanying Example. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLE 1

Efficiency of Nuclear Transfer and Nuclear Addition in Pigs

TABLE 1

Group	Oocytes	Fused (%)	Cleaved (%)	Blastocysts (%)
NT	97	44(45.4)	21(47.7)	6(13.6)
NA	112	54(48.2)	44(81.5)	17(31.5)
Following NT and NA a period of 6 hours was allowed prior to undertaking Activation/Fusion.				
NT: Nuclear transfer NA: Nuclear addition.				

EXAMPLE 2

Efficiency of Nuclear Addition in Pigs and effect of Activation, Fusion and Post Treatment thereon

The nuclear addition of Example 1 was repeated except that a series of modifications were introduced to gauge their impact on efficiency. These included utilising intact MI oocytes in place of MII oocytes, varying the order and arrangement of the activation and fusion steps, utilising chemical inhibitors to prevent co-mingling, and removing or destroying genomic DNA. The results are provided in Table 2.

TABLE 2

Nuclear addition

Oocyte Source	Nuclear manipulation		Post Treatment[1]	Ploidy Correction Intervention	Reconstructed Embryos	Development to Blastocyst (%)
Intact MII oocytes (non enucleated)	Simultaneous Fusion[2] and Activation[3]		Development is allowed to continue without further intervention		360	[illegible] (32)
				[5]Remove or Destroy genomic DNA	120	[illegible] (23)
			1. Cytokinesis Inhibitors (eg Cytochalasin B)		60	[illegible] (35)
			1. Cytokinesis Inhibitors (eg Cytochalasin B)	[5]Remove or Destroy genomic DNA	65	[illegible] (1)
			2. Karyokinesis Inhibitors (eg Colcemid, Nocodazole or Taxol)		57	[illegible] (35)
			2. Karyokinesis Inhibitors (eg Colcemid, Nocodazole or Taxol)	[5]Remove or Destroy genomic DNA	43	[illegible] (1)
			Combination 1 + 2		48	[illegible] (5)
			Combination 1 + 2	[5]Remove or Destroy genomic DNA	36	[illegible] (2)

Oocyte Source	Nuclear manipulation		Post Treatment[1]	Ploidy Correction Intervention	Reconstructed Embryos	Develop[illegible]t to Blastocy[illegible]%)
Intact MII oocytes	Activation[3]	Fusion[2]	Development is allowed to continue without further intervention		126	30 [illegible]
				[5]Remove or Destroy genomic DNA	48	8 [illegible]
Intact MII oocytes	Cell Insertion /Injection (w/out fusion[4])	Activation[3]	Development is allowed to continue without further intervention		53	16 [illegible]
				[5]Remove or Destroy genomic DNA	48	12 [illegible]
			1. Cytokinesis Inhibitors (eg Cytochalasin B)		36	3 [illegible]
			1. Cytokinesis Inhibitors (eg Cytochalasin B)	[5]Remove or Destroy genomic DNA	29	3 [illegible]
			2. Karyokinesis Inhibitors (eg Colcemid, Nocodazole or Taxol)		31	5 [illegible]
			2. Karyokinesis Inhibitors (eg Colcemid, Nocodazole or Taxol)	[5]Remove or Destroy genomic DNA	26	4 [illegible]
			Combination 1 + 2		24	3 [illegible]
			Combination 1 + 2	[5]Remove or Destroy genomic DNA	18	2 [illegible]

Oocyte Source	Nuclear manipulation		Post Treatment[1]	Ploidy Correction Intervention	Reconstructed Embryos	Development to Blastocyst (%)
Intact MI oocytes (non enucleated)	Simultaneous Fusion[2] and Activation [3]		Development is allowed to continue without further intervention		65	
				[5]Remove or Destroy genomic DNA	46	
			1. Cytokinesis Inhibitors (eg Cytochalasin B)		38	
			1. Cytokinesis Inhibitors (eg Cytochalasin B)	[5]Remove or Destroy genomic DNA	27	
			2. Karyokinesis Inhibitors (eg Colcemid, Nocodazole or Taxol)		26	
			2. Karyokinesis Inhibitors (eg Colcemid, Nocodazole or Taxol)	[5]Remove or Destroy genomic DNA	18	
			Combination 1 + 2		23	
			Combination 1 + 2	[5]Remove or Destroy genomic DNA	17	
Intact MI oocytes	Activation[3]	Fusion[2]	Development is allowed to continue without further intervention		42	
				[5]Remove or Destroy genomic DNA	28	

Oocyte Source	Nuclear manipulation		Post Treatment[1]	Ploidy Correction Intervention	Reconstructed Embryos	Development to Blastocyst (%)
Intact MI oocytes	Cell Insertion /Injection (w/out fusion[4])	Activation[3]	Development is allowed to continue without further intervention		43	3
				[5]Remove or Destroy genomic DNA	36	2
			1. Cytokinesis Inhibitors (eg Cytochalasin B)		27	1
			1. Cytokinesis Inhibitors (eg Cytochalasin B)	[5]Remove or Destroy genomic DNA	25	1
			2. Karyokinesis Inhibitors (eg Colcemid, Nocodazole or Taxol)		19	1
			2. Karyokinesis Inhibitors (eg Colcemid, Nocodazole or Taxol)	[5]Remove or Destroy genomic DNA	15	1
			Combination 1 + 2		16	1
			Combination 1 + 2	[5]Remove or Destroy genomic DNA	12	1

Note:

1. Post treatment usually occurs after activation, preferable for 1 hour (or to 12 hours) but would stop prior to normal embryo cleavage and is provided to prevent intermingling of genomic and introduced DNA

2. Fusion is preferred using described Electrical, Viral or PEG methods

3. Activation is preferred using described chemical (Ethanol, Strontium Calcium Ionophore etc) or electrical methods

4. Cell Injection/Insertion is insertion of new DNA (without fusion) using microinjection methods (piezo, microinjection without destruction of the MI or MII plates

5. Removal or destruction of the metaphase DNA is preferred using enucleation (microinjection, piezo etc) or by chemical or laser microsurgery methods. Removal is preferable before the first cleavage or to stop association of metaphase DNA to inserted/fused karyoplast DNA.

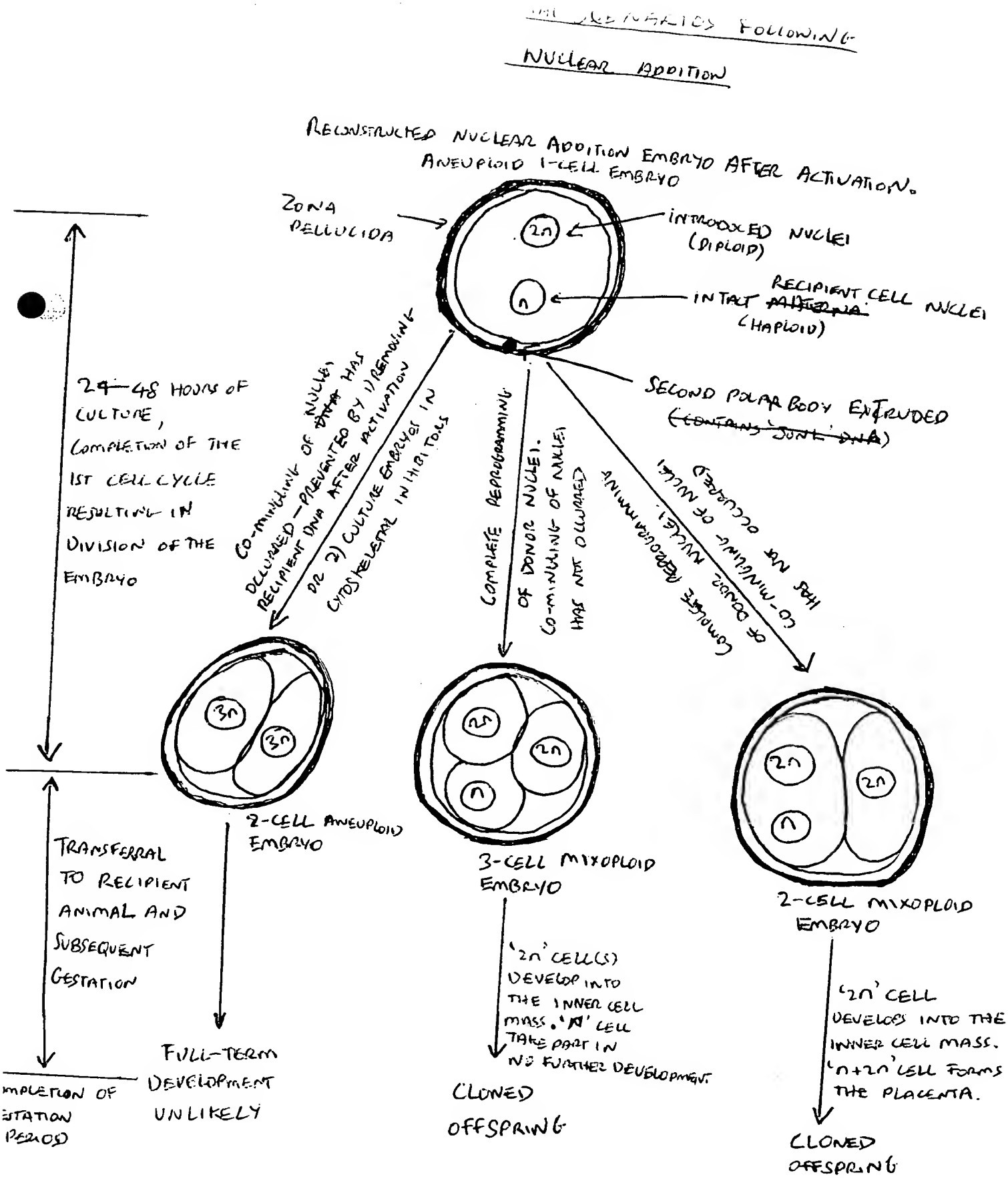
Finally, it is to be understood that various alterations, modifications and/or

as outlined herein.

STEM CELL SCIENCES PTY LTD
By their Registered Patent Attorneys
Freehills Patent Attorneys

30 June, 1999

FIGURE 1


NUCLEAR ADDITION
RECONSTRUCTED NUCLEAR ADDITION EMBRYO AFTER ACTIVATION.
ANEUPLOID 1-CELL EMBRYO
ZONA PELLUCIDA
INTRODUCED NUCLEI (DIPLOID)
INTACT RECIPIENT CELL NUCLEI (HAPLOID)
SECOND POLAR BODY EXTRUDED
(CONTAINS JUNK DNA)
CO-MINGLING OF NUCLEI HAS OCCURRED - PREVENTED BY 1) REMOVING RECIPIENT DNA AFTER ACTIVATION OR 2) CULTURE EMBRYOS IN CYTOSKELETAL INHIBITORS
COMPLETE REPROGRAMMING OF DONOR NUCLEI. CO-MINGLING OF NUCLEI HAS NOT OCCURRED
COMPLETE REPROGRAMMING OF DONOR NUCLEI. CO-MINGLING OF NUCLEI HAS OCCURRED
24-48 HOURS OF CULTURE, COMPLETION OF THE 1ST CELL CYCLE RESULTING IN DIVISION OF THE EMBRYO
2-CELL ANEUPLOID EMBRYO
3-CELL MIXOPLOID EMBRYO
2-CELL MIXOPLOID EMBRYO
TRANSFERRAL TO RECIPIENT ANIMAL AND SUBSEQUENT GESTATION
FULL-TERM DEVELOPMENT UNLIKELY
'2n' CELL(S) DEVELOP INTO THE INNER CELL MASS. 'n' CELL TAKE PART IN NO FURTHER DEVELOPMENT
CLONED OFFSPRING
'2n' CELL DEVELOPS INTO THE INNER CELL MASS. 'n+2n' CELL FORMS THE PLACENTA.
CLONED OFFSPRING